



Evaluation of acute phase proteins, adiponectin and endothelin-1 to determine vascular damage in dogs with heartworm disease (*Dirofilaria immitis*), before and after adulticide treatment

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ABSTRACT

Previous studies have shown the existence of an acute phase response in dogs with heartworm (*Dirofilaria immitis*), probably caused by the vascular inflammation that occurs during the pathogenesis of this disease. In addition, it has been seen that this acute phase response persists after finishing treatment, especially in dogs with pulmonary hypertension (PH). Furthermore, echocardiographic studies have shown that PH and endarteritis appear to persist for at least 10 months after completion of adulticide treatment, suggesting that the vascular changes in these dogs may not be reversible. Therefore, the objective of this study was to evaluate the serum concentrations of different positive acute phase proteins (APP) [C reactive-protein (CRP), haptoglobin and ferritin] and negative APP (albumin and paraoxonase-1 (PON-1)), and the usefulness of the endothelin-1 (ET-1) and adiponectin, in dogs infected by *D. immitis* to evaluate their usefulness as diagnostic biomarkers of vascular damage and PH and their progression throughout therapy up to 7 months after the end of adulticide treatment. Twenty-five heartworm-infected dogs received adulticide treatment, and serum measurements were performed on the day of diagnosis (day 0), day of discharge (day 90), and 6 months after discharge (day 270). In addition, presence or absence of PH was also echocardiographically determined using the Right Pulmonary Artery Distensibility Index. PH was present in 44% of the dogs on day 0 and day 90, and in 48% of dogs on day 270. Alterations were observed in the concentrations of all APP throughout the study, persisting the alterations in PON-1 and ferritin on day 270. Depending on the presence or absence of PH, CRP showed significant differences throughout the study, as did ET-1. On the other hand, adiponectin did not show variations throughout the study, so it did not seem a useful marker in this disease. These results could reflect the possible persistence of vascular inflammation up to 7 months after finishing treatment, whether or not there was PH, and consolidate the study of APP as useful markers in heartworm disease. Moreover, persistent PH could be the consequent clinical manifestation in dogs with more severe vascular alterations so the study of APP, especially CRP, and ET-1 could be especially advantageous in these patients in the early evaluation of the disease, as well as for the determination of disease severity, monitoring therapeutic responses, and predicting outcomes.

1. Introduction

Heartworm disease (*Dirofilaria immitis*) is characterised by the damage caused by the presence of adult worms in the pulmonary arteries and right heart chambers. As soon as they reach their final

location, their presence initiates the production of proliferative endarteritis, causing the onset of vascular remodelling that will chronically produce a series of pathological alterations, mainly pulmonary hypertension (PH) and heart failure (Simón et al., 2012; McCall et al., 2008a, 2008b; Ames and Atkins, 2020) This pathogenic reaction is caused both

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by the presence of adult worms and by the endosymbiont bacterium *Wolbachia pipientis*, which triggers the release of pro-inflammatory and chemotactic cytokines, inducing cell infiltration and amplification of the inflammatory response (Kramer et al., 2008; McCall et al., 2008a, 2008b). PH is one of the most serious complications that can occur in dogs with heartworm disease and for this reason many studies have focused on its diagnosis and assessment, mainly by using diagnostic imaging or serological techniques (Venco et al., 2014b; Carretón et al., 2017; Serrano-Parreño et al., 2017a).

These vascular and inflammatory alterations have also been observed through the existence of an acute phase response in dogs with heartworm (Venco et al., 2014a; Méndez et al., 2014, 2015; Carretón et al., 2017). It was reported that some positive and negative acute phase proteins (APP) were pathologically increased or decreased, respectively, in those dogs with PH, both at the time of diagnosis and at the end of adulticide treatment (Venco et al., 2014a; Carretón et al., 2017). These studies showed the possibility that APP, especially C-reactive protein (CRP), could be used to stage and monitor heartworm disease in dogs and that could also serve as an early biomarker of PH.

Endothelin-1 (ET-1) is a biomarker that has also been shown to be useful in detecting the presence of PH in dogs, both in heartworm disease and in other pathologies (Barman and Isales, 1998; Uchide and Saida, 2005; Hanazono et al., 2016). ET-1 is a powerful vasoconstrictor and promoter of cell proliferation, causing chronic structural changes that lead to cardiopulmonary tissue remodelling and, therefore, a promoter of the development of PH in dogs and humans (Galié et al., 2004; Shao et al., 2011). Furthermore, it has been observed that ET-1 concentrations strongly correlated with the severity of PH (Rubens et al., 2001; Chester and Yacoub, 2014).

Adiponectin is a factor derived from adipocytes that exists in high concentrations in serum and presents systemic vascular anti-inflammatory and protective properties (Ouchi and Walsh, 2007; Villarreal-Molina and Antuna-Puente, 2012). Previous studies have reported that adiponectin has shown an important role in inflammation and remodelling of the pulmonary vasculature and the development of PH in animal models (Matsuda et al., 2002; Medoff et al., 2009; Weng et al., 2010). For this reason, the evaluation of this biomarker is proposed in this study.

Previous studies have shown that PH persists in dogs with heartworm disease, at least up to 10 months after the end of adulticide treatment, demonstrating that this endarteritis may not be reversible after the removal of the parasites (Falcón-Cordón et al., 2019). Thus, given the severity and high prevalence of PH in dogs with heartworm, it would be of interest to carry out more studies focused on the diagnosis and follow-up of PH in these dogs. Therefore, the objective of this study was to evaluate the serum concentrations of different positive APP [CRP, (haptoglobin and ferritin) and negative APP albumin and paraoxonase-1 (PON-1)], as well as ET-1 and adiponectin, in dogs infected by *D. immitis* to evaluate their usefulness as diagnostic biomarkers of vascular damage and PH, and their progression throughout therapy up to 6 months after the end of adulticide treatment.

2. Materials and methods

The study included 25 heartworm-infected dogs living in a hyper-endemic area of *D. immitis* (Montoya-Alonso et al., 2016, 2020). None of the animals had received previous treatment for heartworm infection. A complete record was kept for each animal, including identification (age, sex, and breed), clinical history and demographic data. A complete anamnesis and examination was performed on each patient to rule out the presence of other pathologies that could affect the results; animals with concomitant diseases were excluded from the study. Their body condition was determined based on the nine-scale body condition scoring system, with 1 corresponding to underweight and 9 to obesity (Lafamme, 1997). All were positive to circulating *D. immitis* antigens using a commercial immunochromatographic test kit (Urano test

Dirofilaria®, Urano Vet SL, Barcelona, Spain).

The infected dogs were subjected to adulticide treatment following the treatment protocol recommended by the international Heartworm Societies, applying the recently proposed modifications (European Society of Dirofilariosis and Angiostrongylosis, 2017; American Heartworm Society, 2018; Carretón et al., 2019). In summary, on day 0 the dog is diagnosed and begins the administration of doxycycline (10 mg / kg BID) for 4 weeks and the monthly preventive against heartworm in oral tablets based on ivermectin (≥ 6 mcg/kg of ivermectin and ≥ 5 mg/kg of pyrantel pamoate). On days 30, 60 and 61 the dog is treated with intramuscular doses of melarsomine (2.5 mg / kg). On day 90, the dog is examined and discharged if no abnormalities are found (echocardiographic presence of adult parasites, radiographic abnormalities or cardiorespiratory symptoms). On day 270, 6 months after patient discharge, adulticidal efficacy was confirmed with an antigen detection test. Exercise restriction is recommended throughout treatment, especially from the first dose of melarsomine until the day of discharge.

On the day of diagnosis (day 0), at the end of the treatment (day 90), and 6 months after discharge (day 270), the dogs were subjected to an echocardiographic examination using an ultrasound equipment with spectral and colour Doppler and multi-frequency probes (5.5–10 MHz) (Logic P5, General Electric, New York, United States). The presence or absence of PH was determined using the Right Pulmonary Artery Distensibility Index (RPAD Index) as previously described (Venco et al., 2014b; Visser et al., 2016; Serrano-Parreño et al., 2017a). The presence of visible adult worms in the pulmonary arteries and the right heart chambers was also assessed by echocardiography. Dogs were further evaluated for the presence or absence of microfilariae using a modified Knott test.

Blood was drawn from the cephalic vein of each animal on day 0, day 90, and day 270, to measure CRP, haptoglobin, ferritin, albumin, PON-1, adiponectin, and ET-1. Blood was collected in tubes without anticoagulant, serum was obtained and frozen (-20 °C) until analysis. CRP concentrations were measured using a human immunoturbidimetric test (CRP OSR 6147 Olympus Life and Material Science Europe GmbH, Lismeehan, O'Callaghan Mills, Co. Clare, Ireland), haptoglobin concentrations were measured using a colorimetric method (haptoglobin kit Trideltaphase range, Tridelta Development Ltd.), albumin was determined using a bromocresol green reagent [Albumin OSR 6102 Olympus Life and Material Science Europe GmbH (Irishbranch) Lismeehan, O'Callaghan's Mills, Co. Clare, Ireland], PON-1 activity was determined using p-nitrophenyl acetate as substrate, and ferritin concentrations were measured using an immunoturbidimetric assay with polyclonal anti-human ferritin antibodies (Tina-quant Ferritin, Boehringer Mannheim, Germany). For the determination of adiponectin, the commercially available canine sandwich ELISA (Canine Adiponectin ELISA Kit; Millipore, St. Charles, Missouri, USA) was used. ET-1 was determined by using the kit Endothelin (1–21) EIA (ALPCO Diagnostics, Windham, NH). All techniques had been previously validated for use in dogs (Prosek et al., 2004; Caldin et al., 2009; Martínez-Subiela and Cerón, 2005; Tvarijonavičiute et al., 2010, 2012; Martínez-Subiela et al., 2014).

The results were analysed using the SPSS Base 22.0 software for Windows. The non-parametrical Mann-Whitney U test was used to compare results obtained for dogs negative and positive to heartworm disease and also for the comparison between diseased dogs with PH and without PH. Wilcoxon signed rank test was used to compare the results obtained for the different analytes at three different times (day 0 – day 90 – day 270). In all cases, a p value < 0.05 was determined as significant.

For this study, no ethical approvals were required, since all blood samples were routinely collected for official diagnostic and monitoring purposes and subsequently made available to this study. The study was carried out in accordance with the current Spanish and European legislation on animal protection.

3. Results

Of the dogs that participated in the study, 15 (60%) were female and 10 (40%) were male, with an age between 2 and 10 years (mean: 4.3 years) and an average body condition of 4.3. The modified Knott test showed 12 (48%) dogs with circulating microfilariae. Absence or presence of PH, as well as PH severity, was based on the determination of the RPAD index, and an index < 29% was correlated with PH (Visser et al., 2016). On day 0 of the study, 11 (44%) of the dogs had PH with a mean RPDA index of 21.6%.

All dogs tolerated adulticide treatment with no adverse reactions or showing mild symptoms which did not require treatment or close monitoring. At the end of the treatment (day 90), all the dogs presented absence of microfilariae and no worms were visualised by echocardiography in any of the studied dogs. Animals presented an average body condition of 5.4. Moreover, 11 (44%) presented PH with a mean RPAD index of 21.9%. Six months after discharge (day 270), all dogs had a negative antigen test; furthermore, the absence of microfilariae in the Knott test and adult worms in the echocardiographic study persisted. PH was present in 12 (48%) of them, with a mean RPAD of 22% while their average body condition was 5.2. No significant differences were found in body condition between dogs with PH and dogs without PH on day 0, 90 and 270.

The results obtained in the serological measurements can be seen in Table 1. On day 0 of the study, the concentrations of the APP CRP and ferritin were significantly above the reference values ($p = 0.001$ for both), and the concentrations of albumin and PON-1 were significantly

Table 1
Serum concentrations of the different biomarkers studied, in the total number of dogs evaluated, and divided according to the presence or absence of pulmonary hypertension. Results expressed as mean \pm standard deviation.

Biomarkers	Animals	Day 0	Day 90	Day 270
CRP ($\mu\text{g/mL}$)	Total dogs	18.3 \pm 14.2	7.2 \pm 7.7	4.7 \pm 4.5
	Pulmonary hypertension	24.6 \pm 15.9	10.2 \pm 10	6.5 \pm 5.9
	Normotense	13.3 \pm 10.8	4.9 \pm 4.5	3.1 \pm 1.8
Haptoglobin (mg/dL)	Total dogs	233.2 \pm 153.1	319.1 \pm 131.6	229.4 \pm 159.4
	Pulmonary hypertension	248.1 \pm 158.8	338.3 \pm 126.1	265.9 \pm 169.4
	Normotense	221.6 \pm 153.4	303.9 \pm 138.5	195.7 \pm 148.2
Ferritin ($\mu\text{g/L}$)	Total dogs	242.7 \pm 221.6	186.2 \pm 80.4	170.9 \pm 71.3
	Pulmonary hypertension	274.6 \pm 203.7	178.4 \pm 103.1	153.6 \pm 58.5
	Normotense	217.6 \pm 239.3	192.3 \pm 60.5	186.8 \pm 80.3
PON1 (IU/mL)	Total dogs	1.7 \pm 0.4	1.8 \pm 0.5	1.9 \pm 0.5
	Pulmonary hypertension	1.6 \pm 0.5	1.9 \pm 0.6	2.0 \pm 0.4
	Normotense	1.7 \pm 0.4	1.7 \pm 0.4	1.9 \pm 0.5
Albumin (g/dL)	Total dogs	2.8 \pm 0.3	2.9 \pm 0.4	3.2 \pm 0.5
	Pulmonary hypertension	2.9 \pm 0.3	3.1 \pm 0.3	3.3 \pm 0.2
	Normotense	2.8 \pm 0.3	2.7 \pm 0.6	3.1 \pm 0.7
Adiponectin ($\mu\text{g/L}$)	Total dogs	17.3 \pm 16.4	14.8 \pm 9.4	18 \pm 11.5
	Pulmonary hypertension	15.9 \pm 18.6	14.7 \pm 8.0	16 \pm 8.2
	Normotense	18.3 \pm 15.2	14.8 \pm 10.7	19.8 \pm 14.0
Endothelin (pg/mL)	Total dogs	33.3 \pm 34.6	33.5 \pm 31.7	37.2 \pm 39.3
	Pulmonary hypertension	52.5 \pm 35.1	58.8 \pm 29.5	55.0 \pm 39.5
	Normotense	18.3 \pm 26.6	13.7 \pm 14.6	20.8 \pm 32.5

below the reference values ($p = 0.002$ and $p = 0.000$, respectively). Only the haptoglobin concentrations, although increased, did not differ significantly from the reference values.

Figs. 1 and 2 shows the evolution of the APP studied throughout the study. On day 90, only albumin presented concentrations similar to the reference values; the concentrations of CRP ($p = 0.002$), haptoglobin ($p = 0.009$) and ferritin ($p = 0.002$) remained increased, while the concentrations of PON-1 remained significantly low ($p = 0.000$). When compared to day 0, a marked decrease in CRP and an elevation in haptoglobin were observed ($p = 0.006$ for both); the rest of the APP studied did not show statistically significant differences with respect to the concentrations obtained on day 0.

On day 270 it was observed that ferritin remained above ($p = 0.006$) and PON-1 below ($p = 0.000$) reference values. On the other hand, albumin also appeared significantly increased ($p = 0.014$). When compared to the concentrations obtained on day 90, there were statistically significant differences in the concentrations of albumin ($p = 0.031$) and haptoglobin ($p = 0.023$), while the concentrations of the rest of the APP evaluated remained at similar values.

When the dogs were evaluated according to whether or not they presented PH, it was observed that throughout the entire study, only increased CRP concentrations on day 0 ($p = 0.044$), the day of discharge ($p = 0.038$) and 6 months later ($p = 0.03$), as well as decreased albumin concentrations the day of discharge ($p = 0.038$), showed significant differences between both groups.

On the other hand, adiponectin and ET-1 concentrations did not vary throughout the 3 time point measurements (Fig. 2). Likewise, adiponectin concentrations did not show significant variations between dogs with presence or absence of PH in any of the measurements. However, in the case of ET-1, concentrations were significantly higher in dogs that presented PH on day 0 ($p = 0.001$), day 90 ($p = 0.001$) and day 240 ($p = 0.007$).

4. Discussion

Previous studies have already shown the existence of an acute response in dogs with heartworm disease before beginning the treatment, probably caused by the vascular inflammation that occurs during the pathogenesis of this disease. Thus, previous results showed elevations of the positive APP CRP and decrease of the negative APP PON-1 and albumin (Méndez et al., 2014, 2015). The results obtained in this study support those previously obtained, consolidating the study of APP as useful markers in heartworm disease. In the case of haptoglobin, a paradoxical decrease has always been observed in canine heartworm attributed to the possible presence of haemolytic anaemia (Méndez et al., 2014, 2015); although in this study is not statistically significant, the decrease is still observed.

Ferritin was evaluated for the first time in canine heartworm. The results show that the concentrations of this positive APP were also increased in canine heartworm disease, showing a moderate increase (2–3 times greater than the upper reference value). Ferritin is a ubiquitous iron storage protein and considered as an APP although the precise mechanism underlying increases in serum ferritin is not well understood (Torti and Torti, 2002; Mahroum et al., 2022). Ferritin has been studied in other parasitic diseases, such as *Leishmania*, in which hyperferritinemia was found (Martinez-Subiela et al., 2014; Pardo-Marin et al., 2020).

On the day of discharge, the behaviour of the APPs was similar to previous studies, in which significant changes were observed in CPR and haptoglobin, including the paradoxical increase in haptoglobin at the end of adulticide treatment (Mendez et al., 2015; Carretón et al., 2017) and, except for albumin, values significantly different from the reference values persisted. Regarding ferritin, the increased values persisted. These results show the persistence of vascular inflammation caused by the presence of the parasite; furthermore, thromboembolic processes may still be occurring.

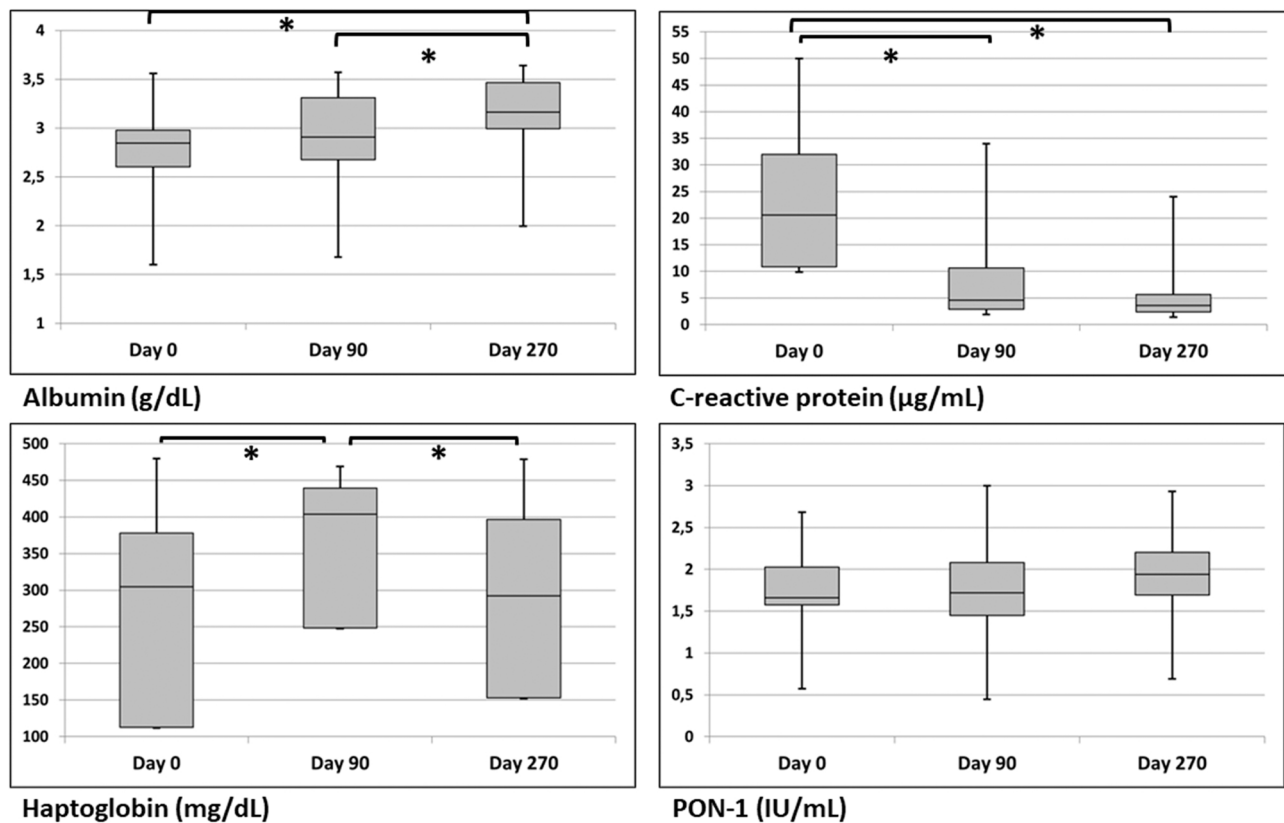


Fig. 1. Serum concentrations of albumin, C-reactive protein, haptoglobin and PON-1 on day 0, day 90 and day 270. The box plots represent median (solid horizontal lines within boxes), 25th and 75th percentiles (boxes) and minimum and maximum values (whiskers). (*): significant differences between groups.

The alterations in ferritin and PON-1 persisted on day 270, indicating that there is still a constant inflammatory component 6 months after the patient is discharged. Noting that remodelled pulmonary arteries often demonstrate inflammation (Farber and Loscalzo, 2005), and that the vascular changes seem to be chronic and not reversible in heartworm-treated dogs (Serrano-Parreño et al., 2017b; Falcón-Cordón et al., 2019), the measurement of APP could be useful to evaluate these patients in the long term. It is the first time that APP have been evaluated in dogs several months after the end of the treatment, so it would be necessary to carry out more studies to determine the repetition of these results.

When the results were evaluated based on the presence or absence of PH, it was observed that CPR appeared significantly higher in dogs with PH, as previously described (Venco et al., 2014a; Carretón et al., 2017). Furthermore, it has also been observed that on the day of discharge, dogs that had PH showed significant increases in CRP concentrations, also previously described (Carretón et al., 2017; Serrano-Parreño et al., 2017b), which is indicative of persistence of the vascular damage one month after the end of the adulticide treatment. Moreover, despite CRP concentrations were within the reference values on day 270, it was observed that the CRP remained significantly elevated in hypertensive dogs.

Adiponectin is a known modulator of pulmonary vascular remodeling (Medoff, 2013), and various studies have shown that there is an enhanced inflammation and vascular remodelling in the vasculature in animal models with adiponectin deficiency, suggesting that adiponectin deficiency could play a role significant in the development of arterial remodelling and PH (Matsuda et al., 2002; Medoff et al., 2009; Weng et al., 2010). However, association between plasma adiponectin levels and PH had not been studied in canine heartworm. Moreover, is an inflammatory cytokine and is involved in the modulation of inflammation and the immune response (Berg and Scherer, 2005) acting as a negative

APP during inflammatory conditions (Tvarijonavičiute et al., 2011). In this study, no difference was observed in adiponectin concentrations between dogs with or without PH, nor significant evolution in time. Since there were no significant differences in the body condition of the dogs with or without PH, the influence of obesity on the results can be ruled out, so the results obtained suggest that this biomarker has limited utility in this disease.

ET-1 concentrations did not vary significantly throughout treatment; however, in all time points a significant increase was detected in dogs with PH. Another previous study determined the usefulness of ET-1 to detect PH in canine heartworm (Uchide and Saida, 2005). The results of this study seem to corroborate the convenience of this biomarker in determining PH, and suggest that ET-1 plays an important role in the pathophysiology of canine heartworm as an aggravating factor by inducing PH. The presence of ET-1 in dogs with heartworm may be the result of the immunological reaction against the parasite, with the release of cytokines, as well as hypoxia and physical stress due to their presence which would consequently cause PH (Barton and Yanagisawa, 2019). As discussed before, previous studies carried out by diagnostic imaging techniques have shown that PH persists in the patient once the parasite has been eliminated (Serrano-Parreño et al., 2017b; Falcón-Cordón et al., 2019); therefore, it is possible that the ET-1 values had remained constant in these animals even after elimination of the parasites. Therefore, this biomarker may be useful in the clinical evaluation of PH in heartworm. To know the true usefulness of this biomarker, it is necessary to carry out more studies assessing this biomarker, and comparing it with reference values in healthy animals.

In conclusion, the results suggest the persistence of vascular damage in dogs in the long term after finishing treatment, so that persistent PH could be the clinical manifestation observed in dogs with more severe vascular alterations (Serrano-Parreño et al., 2017b; Falcón-Cordón et al., 2019). The alterations in some APP confirm that heartworm disease has

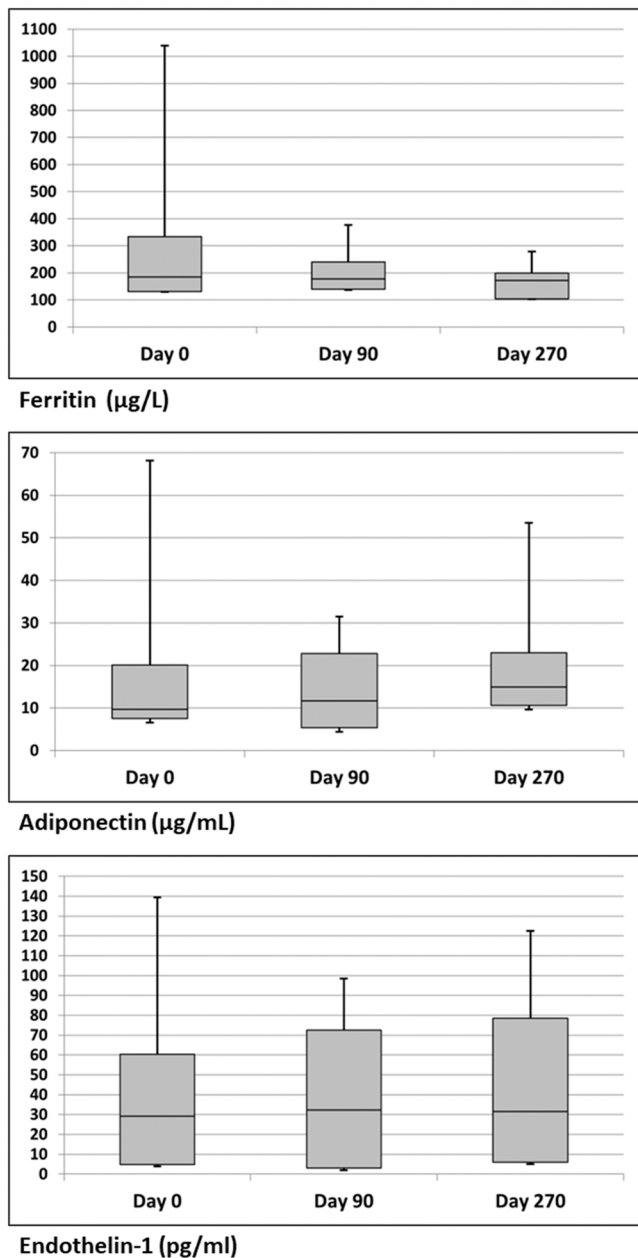


Fig. 2. Serum concentrations of ferritin, adiponectin and endothelin-1 on day 0, day 90 and day 270. The box plots represent median (solid horizontal lines within boxes), 25th and 75th percentiles (boxes) and minimum and maximum values (whiskers). (*): significant differences between groups.

a strong inflammatory component and that part of the inflammatory component of the disease is likely to persist for at least 6 months after discharge, whether or not there is PH. In addition, the persistence of PH previously described by diagnostic imaging techniques is confirmed serologically with the presence of higher levels of endothelin-1 and, possibly, higher concentrations of CRP. So, the clinical use of these biomarkers could play an important role in the early evaluation of the disease, as well as for the determination of disease severity, monitoring therapeutic responses, and predicting outcomes of these patients.

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CRedit authorship contribution statement

Y. Falcón-Cordón: Conceptualization, Methodology, Resources, Writing – original draft. **A. Tvarijonavičute:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision of the research. **J.A. Montoya-Alonso:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision of the research. **A. Muñoz-Prieto:** Conceptualization, Methodology, Resources, Writing – original draft. **A. Caro-Vadillo:** Conceptualization, Methodology, Resources, Writing – original draft, Formal analysis of the data. **E. Carretón:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision of the research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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